DNA sequence and preparation of grass pollen allergen Phl p 4 by recombinant methods

Background of the invention

The present invention relates to the provision of the genetic sequence of the major grass pollen allergen Phl p 4. The invention also covers fragments, new combinations of partial sequences and point mutants having a hypoallergenic action. The recombinant DNA molecules and the derived polypeptides, fragments, new combinations of partial sequences and variants can be utilised for the therapy of pollen-allergic diseases. The proteins prepared by recombinant methods can be employed for the *in vitro* and *in vivo* diagnosis of pollen allergies.

Type 1 allergies are of importance worldwide. Up to 20% of the population in industrialised countries suffer from complaints such as allergic rhinitis, conjunctivitis or bronchial asthma. These allergies are caused by allergens present in the air (aeroallergens) which are liberated from sources of various origin, such as plant pollen, mites, cats or dogs. Up to 40% of these type 1 allergy sufferers in turn exhibit specific IgE reactivity with grass pollen allergens (Freidhoff et al., 1986, J. Allergy Clin. Immunol. 78, 1190-2001).

The substances which trigger type 1 allergy are proteins, glycoproteins or polypeptides. After uptake via the mucous membranes, these allergens react with the IgE molecules bonded to the surface of mast cells in sensitised individuals. If two IgE molecules are crosslinked to one another by an allergen, this results in the release of mediators (for example histamine, prostaglandins) and cytokines by the effector cell and thus in the corresponding clinical symptoms.

A distinction is made between major and minor allergens depending on the relative frequency with which the individual allergen molecules react with the IgE antibodies of allergy sufferers.

In the case of timothy grass (*Phleum pratense*), Phl p 1 (Petersen et al., 1993, J. Allergy Clin. Immunol. 92: 789-796), Phl p 5 (Matthiesen and Löwenstein, 1991, Clin. Exp. Allergy 21: 297-307; Petersen et al., 1992, Int. Arch. Allergy Immunol. 98: 105-109), Phl p 6 (Petersen et al., 1995, Int. Arch. Allergy Immunol. 108, 49-54). Phl p 2/3 (Dolecek et al., 1993, FEBS 335 (3), 299-304), Phl p 4 (Haavik et al., 1985, Int. Arch. Allergy Appl. Immunol. 78: 260-268; Valenta et al., 1992, Int. Arch. Allergy Immunol. 97: 287-294, Fischer et al., 1996, J. Allergy Clin. Immunol. 98: 189-198) and Phl p 13 (Suck et al., 2000, Clin. Exp. Allergy 30: 324-332; Suck et al., 2000, Clin. Exp. Allergy 30: 1395-1402) have hitherto been identified as major allergens.

Phl p 4 has been mentioned as a basic glycoprotein having a molecular weight of between 50 and 60 kDa (Haavik et al., 1985, Int. Arch. Allergy Appl. Immunol. 78: 260-268). The Phl p 4 molecule is trypsin-resistant (Fischer et al., 1996, J. Allergy Clin. Immunol. 98: 189-198), and 70-88% of grass pollen allergy sufferers have IgE antibodies against this molecule (Valenta et al., 1993, Int. Arch. Allergy Immunol. 97: 287-294; Rossi et al., 2001, Allergy 56:1180-1185; Mari, 2003, Clin. Exp. Allergy 33:43-51). Homologous molecules have been described from related grass species (Su et al., 1991, Clin. Exp. Allergy 21: 449-455; Jaggi et al., 1989, Int. Arch. Allergy Appl. Immunol. 89: 342-348; Jaggi et al., 1989, J. Allergy Clin. Immunol. 83: 845-852; Leduc-Brodard et al., 1996, J. Allergy Clin. Immunol. 98: 1065-1072; 14-17). These homologous molecules of the *Poaceae* form allergen group 4, whose molecules have high immunological cross-reactivity with one another both with monoclonal mouse antibodies and with human IgE antibodies (Fahlbusch et al., 1993 Clin. Exp. Allergy 23:51-60; Leduc-Brodard et al., 1996, J. Allergy Clin. Immunol. 98:1065-

1072; Su et al., 1996, J. Allergy Clin. Immunol. 97:210; Fahlbusch et al., 1998, Clin. Exp. Allergy 28:799-807; Gavrović-Jankulović et al., 2000, Invest. Allergol. Clin. Immunol. 10 (6): 361-367; Stumvoll et al. 2002, Biol. Chem. 383: 1383-1396; Grote et al., 2002, Biol. Chem. 383: 1441-1445; Andersson and Lidholm, 2003, Int. Arch. Allergy Immunol. 130: 87-107; Mari, 2003, Clin. Exp. Allergy, 33 (1): 43-51).

In contrast to the above-mentioned major allergens of *Phleum pratense* (Phl p 1, Phl p 2/3, Phl 5a and 5b, Phl p 6 and Phl p 13), the primary structure of Phl p 4 has not yet been elucidated. Likewise, there is no complete sequence of molecules from group 4 from other grass species.

The determination of the N-terminal amino acid sequence was hitherto unsuccessful. However, the causes of this are not known. Fischer et al. (J. Allergy Clin. Immunol., 1996; 98: 189-198) assume N-terminal blocking, but were able to purify an internal peptide after degradation with lysyl endopeptidase and to determine its sequence: IVALPXGMLK (SEQ ID NO 7).

This peptide has homologies to peptide sequences in the ragweed allergens Amb a1 and Amb a2 and similarities to sequences in proteins from maize (Zm58.2), tomato (lat 59, lat 56) and tobacco (G10) (Fischer et al., 1996, J. Allergy Clin. Immunol. 98: 189-198). For *Lolium perenne*, peptide fragments having the following sequence have been described for the basic group 4 allergen: FLEPVLGLIFPAGV (SEQ ID NO 8) and GLIEFPAGV (SEQ ID NO 9) (Jaggi et al., 1989, Int. Arch. Allergy Appl. Immunol. 89: 342-348).

Peptides have likewise been obtained from the group 4 allergen from *Dactylus glomerata* by enzymatic degradation and sequenced:
DIYNYMEPYVSK (P15, SEQ ID NO 10),
VDPTDYFGNEQ (P17, SEQ ID NO 11),
ARTAWVDSGAQLGELSY (P20, SEQ ID NO 12)

and GVLFNIQYVNYWFAP (P22, SEQ ID NO 13) (Leduc-Brodard et al., 1996, J. Allergy Clin. Immunol. 98: 1065-1072).
Peptides have also been obtained from the group 4 allergen of subtropical Bermuda grass (*Cynodon dactylon*) by proteolysis and sequenced:
KTVKPLYIITP (S, SEQ ID NO 14),
KQVERDFLTSLTKDIPQLYLKS (V49L, SEQ ID NO 15),
TVKPLYIITPITAAMI (T33S, SEQ ID NO 16),
LRKYGTAADNVIDAKVVDAQGRLL (T35L, SEQ ID NO 17),
KWQTVAPALPDPNM (P2, SEQ ID NO 18),
VTWIESVPYIPMGDK (V26L, SEQ ID NO 19),
GTVRDLLXRTSNIKAFGKY (L25L, SEQ ID NO 20),
TSNIKAFGKYKSDYVLEPIPKKS (T22L, SEQ ID NO 21),
YRDLDLGVNQVVG (P3, SEQ ID NO 22),
SATPPTHRSGVLFNI (V20L, SEQ ID NO 23),
and AAAALPTQVTRDIYAFMTPYVSKNPRQAYVNYRDLD (V14L, SEQ ID NO 24) (Liaw et al., 2001, Biochem. Biophys. Research Communication 280: 738-743).

However, these described peptide sequences for Phl p 4 and group 4 allergens have hitherto not resulted in the elucidation of the complete primary structure of group 4 allergens.

The object on which the present invention is based therefore comprised the provision of the complete DNA sequence of Phl p 4 and of a corresponding recombinant DNA on the basis of which the Phl p 4 allergen can be expressed as protein and made available for pharmacologically significant utilisation as such or in modified form.

List of figures

Figure 1: Internal DNA sequence (SEQ ID NO 25) of the Phl p 4 gene
Amplicons obtained with genomic DNA were cloned with the degenerated primers No. 30 (sense) and No. 37 (antisense), both shown in italics, and sequenced. The sequence shown represents the consensus from 6 clones. The specific sense primer No. 82 created from this sequence is shown underlined.

Figure 2: 3' end of the nucleic acid sequence (SEQ ID NO 26) of the Phl p 4 gene
Amplicons were obtained with the specific sense primer No. 82 (shown in italics) and an anchor primer in a 3'-RACE PCR with *Phleum pratense* cDNA and sequenced. The sequence shown represents the consensus from 3 sequencing processes and covers the 3' end of the Phl p 4 gene to the stop codon (double underlined). The sequence ranges employed for construction of the antisense primers No. 85 and No. 86 are shown underlined.

Figure 3: Localisation of the Phl p 4 peptides in the deduced amino acid sequence of the Phl p 4 allergen (SEQ ID NO 2)
The peptides P1 - P6 (SEQ ID NO's 27-32) obtained from the amino acid sequencing of the purified and fragmented Phl p 4 allergen can unambiguously be assigned to the amino acid sequence of the Phl p 4 gene derived from the nucleic acid sequence.

Figure 4: Determination of the identity of recombinant Phl p 4 (rPhl p 4) by means of monoclonal antibodies 5H1 (blot A) and 3C4 (blot B) specific for nPhl p 4 by Western blot.
Track 1: *E. coli* total cell extract comprising rPhl p 4 fragment 1-200
Track 2: *E. coli* total cell extract comprising rPhl p 4 fragment 185-500

Track 3: *E. coli* total cell extract comprising rPhl p 4
Track 4: purified nPhl p 4 from *Phleum pratense*
(◀·····): termination or degradation fragments of C-terminal rPhl p 4 fragment or rPhl p 4 entire molecule

Figure 5: Determination of the reactivity of recombinant Phl p 4 (rPhl p 4) using IgE from sera of grass pollen allergy sufferers by Western blot. Extracts of transformed *E. coli* cells which either express the complete Phl p 4 gene or the N-terminal fragment 1-200 or the C-terminal fragment 185-500 were separated in the SDS-PAGE and transferred to nitrocellulose membranes. The blot was incubated with sera from grass pollen-allergic donor A, B or C, and bound IgE was subsequently detected colorimetrically via an anti-human IgE antibody conjugated with alkaline phosphatase.
Track 1: *E. coli* total cell extract comprising rPhl p 4 fragment 1-200
Track 2: *E. coli* total cell extract comprising rPhl p 4 fragment 185-500
Track 3: *E. coli* total cell extract comprising rPhl p 4
Track 4: purified nPhl p 4 from *Phleum pratense*

The numbers used above and below for nucleotide or amino acid sequences "SEQ ID NO" relate to the sequence protocol attached to the description.

Description of the invention

The present invention now provides for the first time the genetic sequence of the major grass pollen allergen Phl p 4, with three dominant sequences (SEQ ID NO 1, 3 and 5) arising from the single nucleotide polymorphisms (SNPs) found.

The present invention therefore relates to a DNA molecule corresponding to a nucleotide sequence selected from a group consisting of SEQ ID NO

1, SEQ ID NO 3 and SEQ ID NO 5 or a DNA molecule corresponding to a nucleotide sequence which encodes for the major allergen Phl p 4 from *Phleum pratense*.

The invention also covers fragments, new combinations of partial sequences and point mutants having a hypoallergenic action.

The invention therefore furthermore relates to corresponding partial sequences, a combination of partial sequences or exchange, elimination or addition mutants which encode for an immunomodulatory, T-cell-reactive fragment of a group 4 allergen of the *Poaceae*.

In addition to the group 4 allergens of the other grass species, the group 13 allergens are also of interest in connection with the present invention since they exhibit a very similar molecular weight to the group 4 allergens in the SDS-PAGE and are difficult to separate by biochemical techniques (Suck et al., 2000, Clin. Exp. Allergy 30: 324-332, Suck et al., 2000, Clin. Exp. Allergy 30: 1395-1402). With the aid of the protein and DNA sequence according to the invention which is now available for the first time, however, it can unambiguously be shown that groups 4 and 13 have significantly different amino acid sequences.

With knowledge of the DNA sequence of naturally occurring allergens, it is now possible to prepare these allergens as recombinant proteins which can be used in the diagnosis and therapy of allergic diseases (Scheiner and Kraft, 1995, Allergy 50: 384-391).

A classical approach to effective therapeutic treatment of allergies is specific immunotherapy or hyposensitisation (Fiebig, 1995, Allergo J. 4 (6): 336-339, Bousquet et al., 1998, J. Allergy Clin. Immunol. 102(4): 558-562). In this method, the patient is injected subcutaneously with natural allergen extracts in increasing doses. However, there is a risk in this method of

allergic reactions or even anaphylactic shock. In order to minimise these risks, innovative preparations in the form of allergoids are being employed. These are chemically modified allergen extracts which have significantly reduced IgE reactivity, but identical T-cell reactivity compared with the untreated extract (Fiebig, 1995, Allergo J. 4 (7): 377-382).

Even more substantial therapy optimisation would be possible with allergens prepared by recombinant methods. Defined cocktails of high-purity allergens prepared by recombinant methods, optionally matched to the individual sensitisation patterns of the patients, could replace extracts from natural allergen sources since these, in addition to the various allergens, contain a relatively large number of immunogenic, but non-allergenic secondary proteins.

Realistic perspectives which may result in reliable hyposensitisation with expression products are offered by specifically mutated recombinant allergens in which IgE epitopes are specifically deleted without impairing the T-cell epitopes which are essential for therapy (Schramm et al., 1999, J. Immunol. 162: 2406-2414).

A further possibility for therapeutic influencing of the disturbed TH-cell equilibrium in allergy sufferers is immunotherapeutic DNA vaccination. This involves treatment with expressable DNA which encodes for the relevant allergens. Initial experimental evidence of allergen-specific influencing of the immune response has been furnished in rodents by injection of allergen-encoding DNA (Hsu et al., 1996, Nature Medicine 2 (5): 540-544).

The present invention therefore also relates to a DNA molecule described above or below or a corresponding recombinant expression vector as medicament.

The corresponding proteins prepared by recombinant methods can be employed for the therapy and for the *in vitro* and *in vivo* diagnosis of pollen allergies.

For preparation of the recombinant allergen, the cloned nucleic acid is ligated to an expression vector, and this construct is expressed in a suitable host organism. After biochemical purification, this recombinant allergen is available for the detection of IgE antibodies by established methods.

The present invention therefore furthermore relates to a recombinant expression vector comprising a DNA molecule described above or below, functionally linked to an expression control sequence and a host organism transformed with the said DNA molecule or the said expression vector.

The invention likewise relates to the use of at least one DNA molecule described above or at least one expression vector described above for the preparation of a medicament for immunotherapeutic DNA vaccination of patients having allergies in the triggering of which group 4 allergens of the *Poaceae* are involved and/or for the prevention of such allergies.

As already stated, the invention can be used as an essential component in a recombinant allergen- or nucleic acid-containing preparation for specific immunotherapy. There are a number of possibilities here. Firstly, the protein with an unchanged primary structure may be a constituent of the preparation. Secondly, through specific deletion of IgE epitopes of the entire molecule or the preparation of individual fragments which encode for T-cell epitopes, a hypoallergenic (allergoidal) form can be used in accordance with the invention for therapy in order to prevent undesired side effects. Finally, the nucleic acid per se, if ligated with a eukaryotic expression vector, gives a preparation which on direct application modifies the allergic immune state in the therapeutic sense.

The invention thus relates to recombinant DNA molecules corresponding to SEQ ID NO 1, 3 or 5, where the nucleotide sequence of positions 1-69 has been derived from the amino acid sequence of the Phl p 4 N-terminus. Codons which frequently occur in *E. coli* were used here. From position 70,

the DNA sequence corresponds to that which has been identified in genomic and cDNA of *Phleum pratense*.
The present invention therefore furthermore relates to a DNA molecule comprising a nucleotide sequence according to SEQ ID NO 1, SEQ ID NO 3 or SEQ ID NO 5, commencing with position 70, which encodes for a polypeptide having the properties of the major allergen Phl p 4 from *Phleum pratense*.

Furthermore, the present invention relates to the polypeptides encoded by one or more of the above-described DNA molecules, preferably in their property as medicament.
These are, in particular, polypeptides according to SEQ ID NO 2, SEQ ID NO 4 or SEQ ID NO 6, where amino acid positions 1-33 have been determined by N-terminal amino acid sequencing of the isolated natural Phl p 4 allergen. Positions 24-500 were derived from the DNA sequence according to SEQ ID NO 1, 3 and 5. Variable amino acids at positions 6, 7, 8 and 9 originate from the N-terminal protein sequencing of various preparations of natural Phl p 4 (Table 1).
Accordingly, the invention also relates to a process for the preparation of polypeptides of this type by cultivation of a host organism according to Claim 11 and isolation of the corresponding polypeptide from the culture.

The invention likewise relates to the use of at least one polypeptide described above for the preparation of a medicament for the diagnosis and/or treatment of allergies in the triggering of which group 4 allergens of the *Poaceae* are involved and for the prevention of such allergies.

These polypeptides or proteins according to the invention which act as allergens for humans are present in the pollen grains of *Phleum pratense*. The pollen grains of the other *Poaceae* species, such as, for example, *Lolium perenne, Dactylis glomerata, Poa pratensis, Cynodon dactylon,*

Holcus lanatus, inter alia, contain homologous allergen molecules (group 4 allergens).
The homology of these molecules has been demonstrated through their immunological cross-reactivity both with murine monoclonal antibodies and also with human IgE antibodies.

Consequently, the invention also relates to sequences which are homologous to the Phl p 4 DNA sequence and corresponding DNA molecules of group 4 allergens from other *Poaceae*, such as, for example, *Lolium perenne, Dactylis glomerata, Poa pratensis, Cynodon dactylon, Holcus lanatus, Triticum aestivum* and *Hordeum vulgare,* which, owing to the sequence homology which exists, hybridise with Phl p 4 DNA under stringent conditions or have immunological cross-reactivity with respect to Phl p 4.

The following procedure was followed in the determination of the protein and DNA sequence of Phl p 4:

The natural allergen Phl p 4 was purified and isolated by described methods (Fahlbusch et al. 1998, Clin. Exp. Allergy 28: 799-807, Suck et al. 2000, Clin. Exp. Allergy 30: 1395-1402). The micropurification and the removal of traces of the group 13 allergen was carried out by the method described by Suck et al. (2000, Clin. Exp. Allergy 30: 1395-1402).
The N-terminal amino acid sequence of this Phl p 4 isolated from *Phleum pratense* was determined by means of Edman degradation. The N-terminal sequences (P1a – f) shown in Table 1 were determined with various batches of Phl p 4. The consensus sequence for the first 15 positions is regarded as being the following sequence: YFPP'P'AAKEDFLGXL (SEQ ID NO 33). Position 14 could not be determined; it is probably occupied by cysteine. The different amino acids in positions 6, 7, 8 and 9 in the different batches indicate variations in the sense of isoforms. Positions 4 and 5 are

occupied by hydroxyproline (P'), which was unambiguously determined by specific analysis in the analyses of preparations p1-a and -b.

Treatment of the SDS-denatured Phl p 4 with the endopeptidase Glu-C (Promega, Heidelberg, Germany) gave various peptides. The amino acid sequences shown in Table 1 were determined for two peptides (P2 and P3). 2 peptides (P4 and P5) were purified by cleavage using the endopeptidase Lys-C (Roche, Mannheim, Germany) and sequenced (Table 1). A further peptide (P6) was isolated by CNBr cleavage and the amino acid sequence was determined (Table 1).

The amino acid sequences of the N-terminal sequence and the internal peptides 2 and 6 were used as the basis for the construction of degenerated primers. Amplicons were prepared with the sense primer No. 30 and the antisense primer No. 37 (Table 2) using genomic DNA from *Phleum pratense*. The clones obtained from these amplicons were sequenced (Fig. 1) and used for the construction of the specific sense primer No. 82 (Table 2). Using a cDNA prepared from the representative mRNA population from *Phleum pratense* pollen and the specific sense primer No. 82 according to the invention and the anchor primer AUAP (Life Technologies, Karlsruhe, Germany), a PCR was carried out under stringent conditions. This approximately 450 kb amplicon was sequenced and the missing sequence as far as the 3' end of the Phl p 4 gene was thus identified (Fig. 2). Based on this C-terminal Phl p 4 sequence determined in accordance with the invention, the specific antisense primers No. 85 and No. 86 were constructed (Table 2). Based on the N-terminal amino acid sequence of the Phl p 4 peptide P1-a (Table 1), the degenerated sense primer No. 29, derived from the DNA encoding for amino acid positions 24-33 (LYAKSSPAYP (SEQ ID NO 34)), was constructed.

A PCR was carried out with primers No. 29 and No. 86 using genomic *Phleum pratense* DNA. This PCR product was employed as the basis for a second PCR (nested PCR) with primers No. 29 and No. 85. The amplicons were inserted into the vector pGEM T-easy (Promega, Heidelberg, Germany), cloned and sequenced. This sequence begins at position 24 calculated from the N-terminus or position 70 of the DNA sequence in accordance with SEQ ID NO 1, 3 or 5 and extends to primer No. 85 (position 1402 in SEQ ID NO 1, 3 or 5), which is localised in the already determined C-terminal section of the Phl p 4 gene. Using these data, the complete amino acid sequence of the Phl p 4 molecule can be constructed from the first 33 amino acid positions, determined by protein sequencing, and the deduced amino acid sequence (477 positions), which can be derived from the clones prepared with primers No. 29/No. 85 and No. 82/anchor primer. The two clones overlap in 197 positions of their nucleotide sequence. The peptide encoded by clone No. 29/No. 85 overlaps in 10 amino acid positions with the N-terminal sequence (positions 1-33), determined by direct amino acid sequencing, of Phl p 4, where the amino acids determined by the two methods correspond.

The amino acid sequence of Phl p 4 based on the directly determined N-terminal amino acids and the deduced amino acid sequence corresponds to the sequences listed in the sequence protocol under SEQ ID NO 2, 4 and 6.

PCR products were prepared with the specific sense primer No. 88 (Table 2) and the specific antisense primer No. 86 both using genomic and using cDNA from *Phleum pratense* and sequenced directly.
This enables PCR errors to be excluded and genetic variations (single nucleotide polymorphisms) to be discovered.
The single nucleotide polymorphisms found for the DNA sequence SEQ ID NO 1 are shown in Table 3. Some of these single nucleotide polymorphisms result in modified amino acids. These are shown in Table 4. Fur-

thermore, DNA clones which result in deviating amino acids with respect to the dominant sequences SEQ ID NO 2, 4 and 6 were sequenced (Table 5). These amino acid variations are to be regarded as isoforms of the Phl p 4 molecule. The existence of such isoforms to be be expected owing to the heterogeneous isoelectric behaviour of natural Phl p 4. All pollen allergens known hitherto have such isoforms. The fact that the DNA fragment determined with primers No. 29 and 86 actually encodes for a protein which is identical with the natural Phl p 4 allergen can also be demonstrated, inter alia, by the fact that homologous peptide sequences in the deduced amino acid sequence of the recombinant Phl p 4 molecule according to the invention are found (Fig. 3) for the identified internal peptides P3, P4 and P5 (Table 1) of natural Phl p 4. The Phl p 4 amino acid sequence described shows that it is a basic molecule having a calculated isoelectric point of 8.99 (SEQ ID NO 2), 8.80 (SEQ ID NO 4) or 9.17 (SEQ ID NO 6), consisting of 500 amino acids. The quantitative amino acid composition is shown in Table 6. The calculated molecular weight of recombinant Phl p 4 is 55.762 (SEQ ID NO 2), 55.734 (SEQ ID NO 4) or 55.624 (SEQ ID NO 6) daltons. This calculated molecular weight agrees very well with the molecular weight of natural Phl p 4 of 55 kDa determined by SDS-PAGE (Fahlbusch et al., 1998, Clin. Exp. Allergy 28: 799 -807 and Suck et al., 2000, Clin. Exp. Allergy 30: 1395-1402).

Molecular weights of between 50 and 60 kDa have also been described for the group 4 allergens of related grass species (Su et al., 1991, Clin. Exp. Allergy 21: 449-455; Jaggi et al., 1989, Int. Arch. Allergy Appl. Immunol. 89: 342-348; Jaggi et al., 1989, J. Allergy Clin. Immunol. 83: 845-852; Leduc-Brodard et al., 1996, J. Allergy Clin. Immunol. 98: 1065-1072; 14 – 17).

For the preparation of the recombinant Phl p 4 protein, the DNA sequence according to SEQ ID NO 1, 3 and/or 5 encoding for Phl p 4 was inserted into expression vectors (for example pProEx, pλCro, pSE 380). For the

N-terminal amino acids known from protein sequencing, *E. coli* optimised codons were used.

After transformation into *E. coli*, expression and purification of the recombinant Phl p 4 by various separation methods, the resultant protein was subjected to a refolding process.
This rPhl p 4 protein obtained in this way gives a single band in the SDS-PAGE which covers the same molecular weight range as natural Phl p 4.
The immunological reactivity of rPhl p 4 has been demonstrated by reaction with the murine monoclonal antibodies 5H1 and 3C4, which had been induced using natural Phl p 4 and cross-react with the homologous proteins (group 4) of the *Poaceae* (Fahlbusch et al., 1998, Clin. Exp. Allergy 28:799-807; Gavrović-Jankulović et al., 2000, Invest. Allergol. Clin. Immunol. 10 (6): 361-367) (Fig. 4). rPhl p 4 reacts with IgE antibodies of allergy sufferers which have demonstrated IgE reactivity with natural Phl p 4. This IgE reactivity and thus the action as allergen has been demonstrated both in the dot test, Western blot and also after adsorption of the allergen on polystyrene microtitre plates. Detection by Western blot is shown in Figure 5. On reaction of rPhl p 4 with basophiles of allergen group 4-reactive grass pollen allergy sufferers, these are stimulated to increased expression of the activation marker CD 203c. This basophile activation by rPhl p 4 clearly shows that this molecule also acts functionally as an allergen.
This rPhl p 4 allergen can thus be employed for the highly specific diagnosis of grass pollen allergy sufferers. This diagnosis can be carried out *in vitro* by detection of specific antibodies (IgE, IgG1-4, IgA) and reaction with IgE-loaded effector cells (for example basophiles from the blood) or *in vivo* by skin test reactions and provocation at the reaction organ.

The reaction of rPhl p 4 with T-lymphocytes of grass pollen allergy sufferers has been detected by allergen-specific stimulation of the T-lymphocytes for proliferation and cytokine synthesis both with T-cells in freshly prepared

blood lymphocytes and on established nPhl p 4-reactive T-cell lines and clones.

Based on the rPhl p 4 DNA sequence described, partial sequences encoding for peptides having from 50 to 350 amino acids were cloned into expression vectors. These partial sequences cover sequentially the complete sequence of rPhl p 4, with overlaps of at least 12 amino acids occurring. The expressed peptides correspond to Phl p 4 fragments. These Phl p 4 fragments do not react individually or as a mixture with the IgE antibodies of allergy sufferers or only do so to a small extent, so that they can be classified as hypoallergenic. In contrast, the mixture of these fragments is capable, in the same way as complete recombinant or natural Phl p 4, of stimulating T-lymphocytes of grass pollen allergy sufferers having Phl p 4 reactivity.

Figure 4 shows as an example the characterisation of two such Phl p 4 fragments corresponding to amino acids 1-200 and 185-500 by binding to Phl p 4-specific monoclonal mouse antibodies. The C-terminal fragment 185-500 reacts only with monoclonal antibody 5H1, while the N-terminal fragment 1-200 clearly reacts with monoclonal antibody 3C4. It can be seen from Figure 5 that fragment 185-500 reacts less strongly with the IgE from the sera of allergy sufferers B and C, i.e. is less allergenic than fragment 1-200, which has reduced IgE reactivity (hypoallergeneity), at least to patient serum C.

The present invention therefore also relates to a DNA molecule described above or below, encoding for a fragment 1-200, with amino acids 1-200 of Phl p 4,

and a DNA molecule encoding for a fragment 285-500, with amino acids 285-500 of Phl p 4.

The triplets encoding for the cysteines were modified by site-specific mutagenesis in such a way that they encode for other amino acids, preferably serine. Both variants in which individual cysteines have been replaced and

those in which various combinations of 2 cysteine radicals or all 5 cysteines have been modified have been prepared. The expressed proteins of these cysteine point mutants have highly reduced or zero reactivity with IgE antibodies of allergy sufferers, but react with the T-lymphocytes of these patients. The present invention therefore furthermore relates to a DNA molecule described above or below in which one, more or all of the cysteine radicals of the corresponding polypeptide have been replaced by another amino acid by site-specific mutagenesis.

The immunomodulatory activity of the hypoallergenic fragments which correspond to polypeptides having T-cell epitopes and those of the hypoallergenic point mutants (for example cysteine polymorphisms) has been demonstrated by reaction thereof with T-cells of grass pollen allergy sufferers.

Such hypoallergenic fragments or point mutants of the cysteines can be employed as preparations for the hyposensitisation of allergy sufferers since they react with equal effectiveness with the T-cells, but, owing to the reduced or entirely absent IgE reactivity, result in reduced IgE-mediated side effects.

If the nucleic acids encoding for the hypoallergenic Phl p 4 variants or the unmodified DNA encoding for Phl p 4 are ligated with a human expression vector, these constructs can likewise be used as preparations for immunotherapy (DNA vaccination).

Finally, the present invention relates to pharmaceutical compositions comprising at least one DNA molecule described above or at least one expression vector described above and optionally further active ingredients and/or adjuvants for immunotherapeutic DNA vaccination of patients having allergies in the triggering of which group 4 allergens of the *Poaceae* are involved and/or for the prevention of such allergies.

A further group of pharmaceutical compositions according to the invention comprises, instead of the DNA, at least one polypeptide described above and is suitable for the diagnosis and/or treatment of the said allergies.

Pharmaceutical compositions in the sense of the present invention comprise, as active ingredients, a polypeptide according to the invention or an expression vector and/or respective pharmaceutically usable derivatives thereof, including mixtures thereof in all ratios. The active ingredients according to the invention can be brought here into a suitable dosage form together with at least one solid, liquid and/or semi-liquid excipient or adjuvant and optionally in combination with one or more further active ingredients.
Particularly suitable adjuvants are immunostimulatory DNA or oligonucleotides having CpG motives.

These compositions can be used as therapeutic agents or diagnostic agents in human or veterinary medicine. Suitable excipients are organic or inorganic substances which are suitable for parenteral administration and do not adversely affect the action of the active ingredient according to the invention. Particularly suitable for parenteral administration are solutions, preferably oil-based or aqueous solutions, furthermore suspensions, emulsions or implants. The active ingredient according to the invention may also be lyophilised and the resultant lyophilisates used, for example, for the preparation of injection preparations. The compositions indicated may be sterilised and/or comprise adjuvants, such as lubricants, preservatives, stabilisers and/or wetting agents, emulsifiers, salts for modifying the osmotic pressure, buffer substances and/or a plurality of further active ingredients.
Furthermore, sustained-release preparations can be obtained by corresponding formulation of the active ingredient according to the invention.

The invention thus also serves for improving *in vitro* diagnosis as part of allergen component-triggering identification of the patient-specific sensitisation spectrum. The invention likewise serves for the preparation of significantly improved preparations for the specific immunotherapy of grass pollen allergies.

Table 1 Amino acid sequence of Phl p 4 peptides

Preparation	Peptide batch	SEQ ID NO	Amino acids 1 6 11 16 21 26 31
Intact Phl p 4	P1-a	35	YFPP'P' AAKED FLGXL VKEIP PRLLY AKSSP AYP
	P1-b	36	YFPP'P' AAKED FLGXL VKE-P PRLLY AKSSP
	P1-c	37	YFPXX AAKED FLGXL
	P1-d	38	YFPXX AKKED FLGXL
	P1-e	39	YFPXX AAKDD FLGXL
	P1-f	40	YFPXX LANED F
Glu-C fragments	P2	41	SATPF XHRKG VLFNI QYV
	P3	42	GLXYR XLXPE
Lys-C fragments	P4	43	KXMGD DHFXA VR
	P5	44	APEGA VDI I
CNBr fragment	P6	45	MEPYV SINPV QAYAN Y

Table 2 Degenerated and specific sense and antisense primers constructed on the basis of Phl p 4 peptide sequences and DNA sequences

Primer No.	Peptide/ DNA	Sense/ anti-sense	SEQ ID NO	Nucleotide sequence
29	Phl p 4-P1	s	46	YTN TAY GCN AAR WSN WSN CCN GCN TAY CC
30	Phl p 4-P2	s	47	CAY MGN AAR GGN GTN YTN TTY AAY ATM C
37	Phl p 4-P6	as	48	TAR TTN GCR TAN GCY TGN ACN GGR TT
82	Phl p 4-DNA-NYW	s	49	ACT ACT GGT TCG CCC CGG GAG CC
85	Phl p 4-DNA-GLV	as	50	TGA AGT ATT TCT GGC CCC ACA CCA AAC C
86	Phl p 4-DNA-QRL	as	51	CCC TTG GTG ATG GCG AGC CTC

				TGG
88	Phl p 4-DNA-PSV	s	52	CTC AGT CCT GGG GCA GAC CAT CC

The nucleotide sequences of primers 82, 85, 86 and 88 is shown in the usual 4-letter code. In the case of primers 29, 30 and 37, the IUPAC-IUB DNA code is used; the letter 'N' here stands for inosine.

Table 3 Detected single nucleotide polymorphisms

Position in sequence	Nucleotide according to SEQ ID NO 1	Detected SNPs
85	T	A
130	C	A
159	G	A
160	A	C
169	G	A
185	C	T
186	C	A
222	G	C
226	G	A
227	G	C
228	T	C
237	C	T
273	C	T
285	C	T
286	C	T
298	G	A
299	A	C
303	C	T
309	C	G
318	T	C
320	G	A
333	C	G
348	G	C
369	C	G
409	C	T
411	C	T
420	T	C
421	A	C
423	A	C
424	G	A

425	T	C
456	C	G
462	C	A
522	G	C
525	C	G
567	G	A
618	C	T
655	A	C
657	G	A
662	G	A
680	C	T
684	G	C
690	C	A
691	G	A
693	G	A
703	C	T, A
710	A	C
711	G	A
713	C	T
743	G	A
750	G	A
768	C	T
773	A	C
790	G	A
798	G	C
801	G	A
804	C	G
809	C	A
834	G	C
844	C	A
859	A	T
865	A	G
879	G	C
895	G	C
900	G	C, A
918	G	A
961	A	G
962	A	C
964	A	C
987	G	C
994	A	T
1020	G	A
1023	G	C
1036	G	C
1040	C	T
1041	G	C

1047	C	A
1051	A	G
1052	G	A, C
1053	G	A, C, T
1056	G	C
1069	T	C
1073	G	A
1084	C	G
1086	G	C
1090	C	T
1098	G	C
1151	G	C
1152	G	C
1155	G	C
1161	G	C
1185	C	G
1229	G	C
1233	G	C
1239	A	C
1240	T	C
1242	G	C
1257	G	C
1266	C	T
1269	C	T
1278	A	C, G
1305	C	G
1308	C	T
1311	C	A
1335	G	C
1350	G	C
1357	T	A
1359	A	G
1370	G	C
1377	T	C
1378	T	A
1379	T	A
1383	G	C
1398	C	T
1411	T	C
1414	C	G
1425	C	A
1428	C	T
1443	G	C
1449	C	T
1464	G	A
1485	G	A

1498	A	C

Table 4 Amino acid exchanges as a consequence of single nucleotide polymorphisms

Position in sequence	Amino acid according to SEQ ID NO 2	Detected exchanges
6	A	L
7	A	K
8	K	N
9	E	D
29	S	T
54	I	L
57	V	I
62	A	V
76	G	T, N, S
100	E	T
107	S	N
137	H	Y
141	T	P
142	V	A, T
189	T	K
219	K	Q
221	R	K
227	P	L
231	V	I
235	P	T, S
237	K	T
238	A	V
248	R	K
258	D	A
264	V	I
270	T	K
282	Q	K
287	M	L
289	S	G
299	A	P
321	N	A
322	I	L
332	T	S
346	E	Q
347	P	L

351	R	E, T
357	F	L
358	S	N
362	L	V
364	P	S
384	W	S
410	G	A
419	E	D
456	F	Y
457	S	A, N
460	L	K
468	K	M
472	Q	E
498	K	Q

Table 5 Deviating amino acid positions in individual recombinant Phl p 4 clones compared with SEQ ID NO 2

Example	Deviating positions*
Clone 1	L54, I57, V62, S76, T100, N107, Y137, P141, T142, K189, Q219, K221, L227, I231, S235, T237, V238, K248, A258, I264, K270, K282, L287, P299, A321, L322, S332, Q346, P347, T351, L357, N358, V362, S384, A410, D419, Y456, A457, K460, E472
Clone 2	L54, I57, V62, T76, T100, N107, Y137, P141, T142, K189, Q219, K221, I231, S235, T237, V238, K248, A258, I264, K270, K282, L287, P299, A321, L322, S332, Q346, P347, T351, L357, N358, V362, S384, A410, D419, Y456, A457, K460, E472
Clone 3	P141, K282, L287, P299, L347, E351
Clone 4	G289, A410, D419, Y456, A457, K460, E472
Clone 5	L347, E351, S384, A410, D419, Y456, A457, K460, E472
Clone 6	N107, Y137, P141, T142, K189, Q219, K221, I231, S235, T237, V238, K248, A258, I264, K270, K282, L287, P299, A321, L322, S332, Q346, P347, T351, L357, N358, V362, S384, A410, D419, Y456, A457, K460
Clone 7	K248, A258, I264, K270, K282, L287, P299, A321, L322, S332, Q346, P347, T351, L357, N358, V362, S384
Clone 8	Q219, K221, I231, S235, T237, V238, K248, A258, I264, K270, K282, L287, P299, E351
Clone 9	M231, T246, A251, C263, G289, L307, L309, E334
Clone 10	Q219, K221, I231, S235, T237, M238, V242, V246, K248, A258, I264, K270, K282, L287, P299, A321, L322, S332, Q346,

	P347, T351, N358, V362, S384, insertion of GA between positions 407 and 408, N452, Y456, A457, K460, E472
Clone 11	Insertion of GA between positions 407 and 408

*[Amino acid according to SEQ ID NO 2 / position in sequence / deviating amino acid]

Table 6 Amino acid composition of Phl p 4

Amino acids	Number	% by weight
Charged	138/138/138	33.89/33.86/33.93
Acid	45/46/43	9.82/10.05/9.38
Basic	54/53/55	13.67/13.39/13.78
Polar	120/119/124	24.88/24.71/25.89
Hydrophobic	180/180/180	35.64/35.66/35.43
A Ala	40/40/41	5.10/5.10/5.24
C Cys	5/5/5	0.92/0.93/0.93
D Asp	24/24/24	4.95/4.96/4.97
E Glu	21/22/19	4.86/5.10/4.41
F Phe	24/24/22	6.33/6.34/5.82
G Gly	42/42/40	4.30/4.30/4.10
H His	10/10/9	2.46/2.46/2.22
I Ile	29/29/30	5.88/5.89/6.10
K Lys	29/29/33	6.67/6.67/7.60
L Leu	33/33/35	6.70/6.70/7.12
M Met	11/11/10	2.59/2.59/2.36
N Asn	22/22/23	4.50/4.50/4.72
P Pro*	38/39/39	6.62/6.80/6.81
Q Gln	15/15/15	3.45/3.45/3.46
R Arg	25/24/22	7.00/6.73/6.18
S Ser	32/32/33	5.00/5.00/5.17
T Thr	22/21/22	3.99/3.81/4.00
V Val	41/41/40	7.29/7.29/7.13
W Trp	13/13/12	4.34/4.34/4.02
Y Tyr	24/24/26	7.02/7.03/7.63

* including hydroxyproline

The values are given for the three dominant sequences in the order SEQ ID NO 2 / SEQ ID NO 4 / SEQ ID NO 6.